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Role of Sec61α in the Regulated Transfer of the Ribosome–Nascent Chain Complex from the Signal Recognition Particle to the Translocation Channel

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Summary

Targeting of ribosome–nascent chain complexes to the translocon in the endoplasmic reticulum is mediated by the concerted action of the signal recognition particle (SRP) and the SRP receptor (SR). Ribosome-stripped microsomes were digested with proteases to sever cytoplasmic domains of SRα, SRβ, TRAM, and the Sec61 complex. We characterized protein translocation intermediates that accumulate when Sec61α or SRβ is inactivated by proteolysis. In the absence of a functional Sec61 complex, dissociation of SRP54 from the signal sequence is blocked. Experiments using SR proteoliposomes confirmed the assembly of a membrane-bound posttargeting intermediate. These results strongly suggest that the Sec61 complex regulates the GTP hydrolysis cycle of the SRP-SR complex at the stage of signal sequence dissociation from SRP54.

Introduction

Protein translocation across the mammalian endoplasmic reticulum is mediated by a surprisingly small number of protein components that cooperate to accomplish the selective attachment of a ribosome–nascent chain complex (RNC) to a protein-conducting channel in the rough endoplasmic reticulum (RER) (as reviewed by Walter and Johnson, 1994; Rapoport et al., 1996). The 54 kDa subunit of the signal recognition particle (SRP) binds to the hydrophobic core of the signal sequence as the nascent polypeptide emerges from the large ribosomal subunit. Selective targeting of the SRP-RNC to the RER is driven by the interaction between SRP and the SRP receptor (SR), a heterodimeric membrane protein that is restricted to the RER. The interaction between the SR and the SRP-RNC results in the GTP-dependent dissociation of the nascent polypeptide from SRP54 (Connolly et al., 1991) and transfer of the signal sequence to Sec61α. The GTPase cycles of SRP54 and SRα are obligatorily coupled (Connolly and Gilmore, 1993; Powers and Walter, 1995), as neither SRP54 nor SRα stably binds GTP prior to the assembly of an SRP-SR complex (Rapiejko and Gilmore, 1997). GTP binding to SRP54 is thought to reduce the affinity between SRP54 and the signal sequence (Miller et al., 1993), while GTP binding to SRα enhances the affinity between SRP and the SR (Rapiejko and Gilmore, 1997). GTP hydrolysis allows dissociation of the SRP-SR complex (Connolly et al., 1991). One role for SRβ is to provide a membrane binding site for the SRα subunit (Young et al., 1995). However, the membrane anchor of *Saccharomyces cerevisiae* SRβ is dispensable in vivo suggesting that membrane attachment of the SR via SRβ is not crucial for SR function (Ogg et al., 1998). A recent report suggests that RNCs bind to SRβ in a GTP-dependent manner resulting in hydrolysis of GTP by SRβ (Bacher et al., 1999).

The heterotrimeric Sec61 complex forms the structural core of the protein translocation channel (Görlich et al., 1992). As visualized by electron microscopy, the channel consists of a quasipentagonal oligomeric pore composed of three to four Sec61 heterotrimers (Hanein et al., 1996). A three-dimensional image reconstruction of a ribosome bound to the yeast Sec61 complex reveals a single point of attachment that aligns the translocation channel with the ribosome exit site for the nascent polypeptide (Beckmann et al., 1997). Nascent polypeptides contact Sec61α at all stages of transport across the RER (Mothes et al., 1994); hence, the transmembrane (TM) spans of Sec61α form the hydrophilic protein-conducting channel across the RER that has been detected by biophysical techniques (Simon and Blobel, 1991; Crowley et al., 1993). In addition to the SR and the Sec61 complex, SRP-dependent translocation of most polypeptides across reconstituted proteoliposomes requires the integral membrane protein TRAM (Görlich and Rapoport, 1993), which is thought to be a peripheral component of the translocon. One role of TRAM in protein translocation is to facilitate insertion of the nascent chain into the Sec61 channel following the initial interaction between the signal sequence and Sec61α (Voigt et al., 1996).

Sec61 channels that are not engaged in translocation are likely occluded by nontranslating ribosomes, as the channel has a high affinity for the ribosome (Kalies et al., 1994). Nonetheless, 80S ribosomes do not compete with SRP-RNC complexes for attachment to the translocon (Neuhof et al., 1998; Raden and Gilmore, 1998), suggesting that a dormant ribosome can be displaced from the Sec61 complex by an SRP-RNC. Efficient transfer of an RNC from the SRP-SR complex to the translocation channel may necessitate a mechanism that inhibits signal sequence dissociation from SRP54 if the adjacent Sec61 complex is occupied by a translating ribosome. To test this hypothesis, we sought conditions that would cause the accumulation of translocation intermediates that precede RNC transfer from SRP to the Sec61 complex. Digestion of ribosome-stripped microsomes severs SRα, TRAM, Sec61α, and Sec61β. Reconstitution of Sec61 complex–deficient membranes with SRα yielded microsomes that were defective in SRP-dependent translocation and accumulated a GTP-stabilized complex between the SR and an SRP-RNC. A GTP-stabilized intermediate was also formed when SRP-RNCs were incubated with SR proteoliposomes. We propose that the GTP hydrolysis cycle of the SRP-SR complex is regulated by the Sec61 complex at the level of signal sequence dissociation from SRP54.

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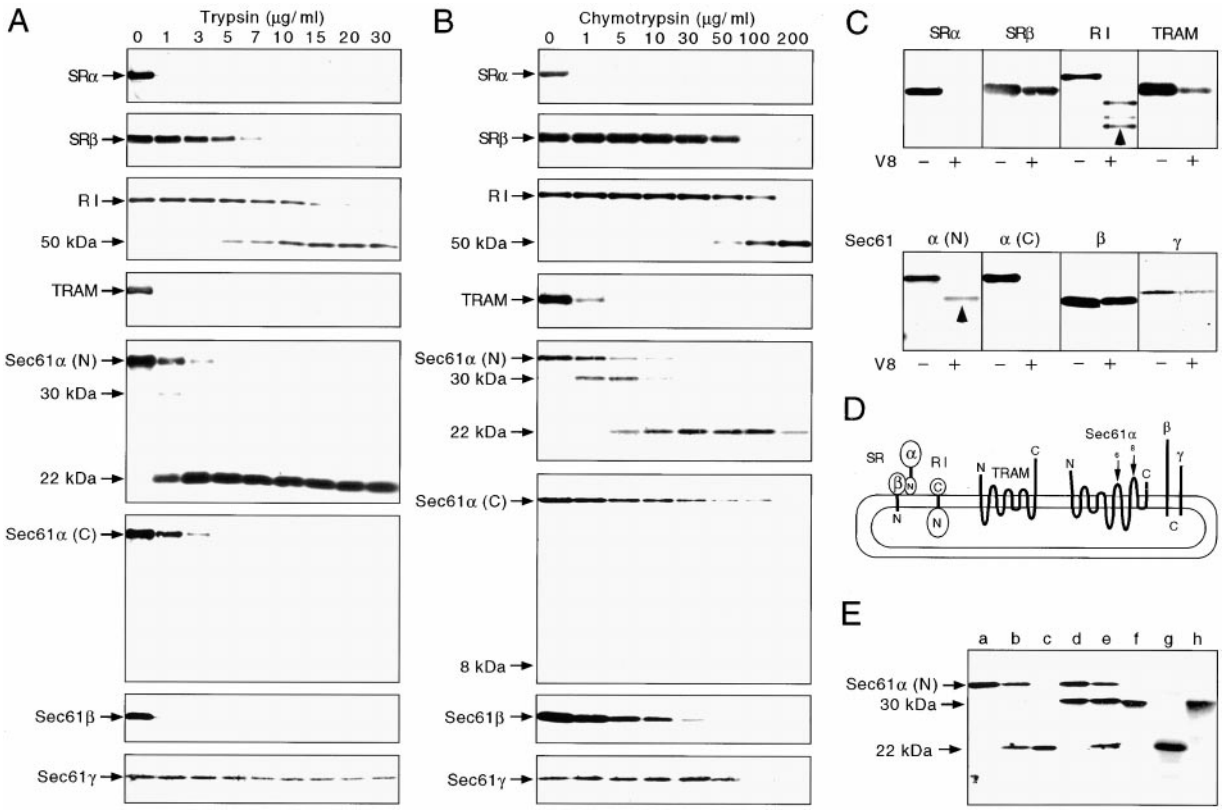

Figure 1. Protein Immunoblot Analysis of Protease-Digested PK-RM

Aliquots of the T_X-PK-RM (A), C_X-PK-RM (B), and V_{200}-PK-RM (C) were resolved by PAGE in SDS, transferred to PVDF membranes, and probed with antibodies to SRα, SRβ, ribophorin I (RI), TRAM, the N terminus of Sec61α [Sec61α (N)], the C terminus of Sec61α [Sec61α (C)], Sec61β, and Sec61γ. In (B), a darker exposure of the Sec61α (C) blot is shown to visualize the 8 kDa C-terminal fragment. The apparent molecular weight of Sec61α and ribophorin I digestion products was estimated by comparison with molecular weight markers. In (C), the 50 kDa ribophorin I fragment and the 30 kDa Sec61α fragment are marked by arrowheads.

(D) The topologies of SRα, SRβ, ribophorin I, TRAM, Sec61α, Sec61β, and Sec61γ in the ER membrane are shown. The relative size of the cytosolic and lumenal tails and loops of the Sec61 subunits and the TRAM protein are shown.

(E) Protease-digested PK-RM were resolved by PAGE in SDS adjacent to ^{35}S methionine-labeled Sec61α274 and Sec61α393. The following PK-RM samples were analyzed: (a) T_0, (b) T_1, (c) T_{10}, (d) C_1, (e) C_5, (f) V_{200}, (g) Sec61α274, and (h) Sec61α393. After the ECL image was obtained, the PVDF membrane was exposed to X-ray film to detect Sec61α274 and Sec61α393.

Results

Proteolysis of the SR, TRAM, and the Sec61 Complex in Ribosome-Stripped Microsomes

Proteases were used to sever the cytoplasmic domains of protein translocation components to obtain microsomes that were defective in protein translocation. To enhance protease access to the Sec61 complex (Kalies et al., 1994), the microsomes were incubated with puromycin and extracted with high salt to detach ribosomes from the translocon. The puromycin-high salt extracted rough microsomes (PK-RM) were digested with trypsin, chymotrypsin, or endoproteinase Glu-C (see the Experimental Procedures) and are designated respectively as T_X-PK-RM, C_X-PK-RM, and V_X-PK-RM, where the subscript X denotes the concentration of protease, in μg/ml, used for the digestion.

The topologies of the SRP receptor, ribophorin I, the TRAM protein, and the Sec61 complex are diagrammed in Figure 1D. Protein immunoblot analysis using SRα and SRβ-specific antibodies revealed that SRα was far more sensitive to digestion than SRβ (Figures 1A–1C). Trypsin, chymotrypsin, and elastase sever SRα between an N-terminal domain that binds to SRβ and the C-terminal 52 kDa segment that contains the GTPase domain (Lauffer et al., 1985). The 50 kDa lumenal domain of ribophorin I (RI), an oligosaccharyltransferase subunit, was the limit digestion product demonstrating that the microsomes remain impervious to the proteases (Figures 1A–1C). Intact TRAM protein was not present in the T_1-PK-RM (Figure 1A); traces of TRAM were detected in C_1-PK-RM (Figure 1B), while 30% of the TRAM protein was not digested by endoproteinase Glu-C (Figure 1C). Immunoreactive membrane-bound fragments of TRAM were not produced by cleavage with either trypsin or chymotrypsin. The TRAM protein has eight predicted membrane-spanning segments with both the N and C termini facing the cytoplasm (Figure 1D). As the antibody to TRAM was raised against a synthetic peptide corresponding to the TRAM C terminus, loss of TRAM immunoreactivity cannot be equated with extensive degradation of TRAM.

The cytoplasmic face of the 476-residue Sec61α subunit consists of the N and C termini plus the even-numbered loops that connect the ten experimentally verified TM segments (Wilkinson et al., 1996). The antibody

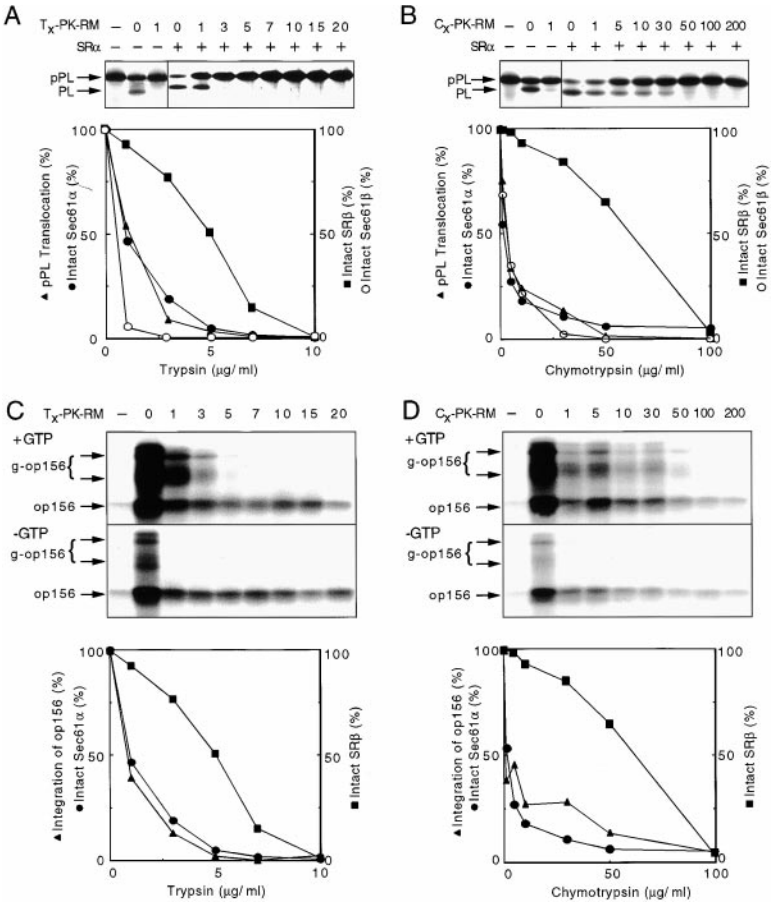

Figure 2. Translocation Activity of the Protease-Digested PK-RM

T_X-PK-RM (A) or C_X-PK-RM (B) were assayed for translocation of preprolactin. The 25 μl reticulocyte lysate translations contained 1.2 eq of T_X-PK-RM or C_X-PK-RM and, as noted, were supplemented with the 52 kDa SRα fragment (200 fmol). Preprolactin (pPL) and prolactin (PL) were quantified after PAGE in SDS. (C and D) Assays of op156 integration contained 1.2 eq of T_X-PK-RM (C) or C_X-PK-RM (D) and 200 fmol of the SRα fragment and were conducted in the presence (upper panels) or absence (lower panels) of GTP. Membrane pellets obtained after alkaline sucrose gradients were analyzed by PAGE in SDS to resolve glycosylated op156 (g-op156) from nonglycosylated op156. SRP-dependent membrane integration of op156 (op156 + g-op156) was calculated after subtracting as background the quantity of op156 (op156 + g-op156) that was integrated in the absence of GTP. Preprolactin translocation (triangles) across T_X-PK-RM (A) or C_X-PK-RM (B) and SRP-dependent op156 integration (triangles) into T_X-PK-RM (C) or C_X-PK-RM (D) is expressed relative to the translocation/integration activity of T_0-PK-RM and C_0-PK-RM assayed in the presence of the SRα fragment. The percentages of intact SRβ (squares), Sec61α (filled circles), and Sec61β (open circles) in the T_X-PK-RM and C_X-PK-RM was quantified by scanning the ECL images shown in Figure 1. For Sec61α, the plotted data is the average value obtained by scanning blots probed with the N- and C-terminal antibodies.

raised against the N terminus of Sec61α recognized 22 and 30 kDa digestion products (Figures 1A, 1B, and 1C). To determine which loops in Sec61α were severed, we prepared N-terminal segments of Sec61α by in vitro translation of truncated Sec61α mRNAs. The 22 and 30 kDa Sec61α fragments comigrate with Sec61α translation products that terminate at Arg-274 (loop 6) and Lys-393 (loop 8), respectively (Figure 1E). The C-terminal-specific antibody did not recognize membrane-bound tryptic fragments derived from Sec61α, indicating that the C terminus of Sec61α is cleaved at Lys-463. In contrast, a C-terminal 8 kDa fragment of Sec61α was detected in the $C_{1\text{-}10}$-PK-RM (Figure 1B), which is derived by cleavage within loop 8. Decreases in N-terminal immunoreactivity were caused by digestion with high concentrations of chymotrypsin (i.e., C_{200}-PK-RM) or endoproteinase Glu-C. Sec61β (96 residues) and Sec61γ (68 residues) each have a single predicted TM span located near the C terminus (Figure 1D). Sec61β was quite sensitive to digestion by trypsin (Figure 1A), less sensitive to chymotrypsin (Figure 1B), and essentially resistant to endoproteinase Glu-C (Figure 1C). Sec61γ was the least protease-sensitive subunit of the Sec61 complex (Figures 1A, 1B, and 1C).

Proteolysis-Induced Translocation Defects

Severing the cytoplasmic domain of a translocation component could have no effect on translocation activity or could reduce translocation activity by inactivating the protein or by producing an inhibitory fragment. To simplify the analysis of the protease-digested PK-RM, translocation activity was assayed using the TRAM-independent substrate preprolactin (Voigt et al., 1996). The protease-digested microsomes were first assayed for translocation activity in the absence of SRα (Figures 2A and 2B, left three lanes). Unlike the mock digested membranes, the T_1-PK-RM and C_1-PK-RM were unable to translocate and process preprolactin. The SR in the protease-digested PK-RM was reconstituted by the addition of the purified 52 kDa SRα fragment that contains the GTPase domain. A 2-fold molar excess of the SRα fragment was added relative to the SR content of PK-RM to insure that SRα was not the limiting component. Protein immunoblot experiments showed that each of the membrane preparations (except the C_{200}-PK-RM) bind quantities of the SRα fragment that are similar to the endogenous SRα content (~100 fmol/eq) of the undigested PK-RM (data not shown). As observed previously for trypsin-digested K-RM (Gilmore et al., 1982), the SRα fragment can partially reconstitute the translocation activity of the T_1-PK-RM (Figure 2A). More extensive digestion of the PK-RM with trypsin (e.g., T_5-PK-RM) resulted in a translocation defect that could not be reconstituted with exogenous SRα. Likewise, the SRα fragment could reconstitute the translocation activity of the less extensively digested C_X-PK-RM (Figure 2B) but could not reconstitute the activity of the C_{50}-PK-RM (Figure 2B) or the V_{200}-PK-RM (data not shown).

Based upon the hypothesis that proteolysis of the Sec61 complex or SRβ might be responsible for the

translocation defect, we compared the SRα-dependent translocation activity to the membrane content of intact SRβ, Sec61α, and Sec61β (Figures 2A and 2B). Proteolysis of SRβ (squares) and Sec61γ (data not shown) did not correlate with the protease-mediated loss in translocation activity (triangles). In contrast, digestion of Sec61α (filled circles) correlated closely with loss of translocation activity, suggesting that proteolysis of Sec61α may be responsible for the defect in SRP-dependent translocation of preprolactin. Digestion of Sec61β by chymotrypsin (Figure 2B), but not trypsin (Figure 2A), correlated with the protease-mediated inhibition of translocation activity.

Sec61β is required for efficient cotranslational translocation but is dispensable when the nascent polypeptide is tethered to the ribosome as a translation-arrested peptidyl-tRNA (Kalies et al., 1998). While proteolysis of Sec61β may contribute to the defect in translocation of preprolactin, it should not influence translocation or integration of a translation-arrested substrate. To address this possibility, we analyzed membrane integration of op156, a 156-residue opsin nascent chain that is produced by translation of a truncated opsin mRNA (Figures 2C and 2D). The protease-digested PK-RM were reconstituted with the SRα fragment and assayed in the presence or absence of GTP to discriminate between SRP-dependent and SRP-independent integration of op156. Integration of op-156 into the PK-RM can occur by an SRP and GTP-independent integration mechanism that is analogous to that described for other translation-arrested polypeptides (Wiedmann et al., 1994; Jungnickel and Rapoport, 1995; Raden and Gilmore, 1998). GTP-dependent integration of op156 into the undigested PK-RM was accompanied by oligosaccharide addition to one or both of the glycosylation sites to yield g-op156 (Figures 2C and 2D, upper panels). SRP-independent integration of op156 yielded substantially reduced amounts of g-op156 and slightly reduced amounts of op-156 (lower panels). Whereas both targeting pathways can occur in the presence of GTP, subtraction of the GTP-independent signal yields a reliable estimate of the SRP-dependent integration of op156. In the absence of the SRα fragment, glycosylation of op156 by the T_1-PK-RM and the C_1-PK-RM was undetectable (data not shown). The addition of the SRα fragment to the protease-digested PK-RM resulted in a partial restoration of g-op156 synthesis as quantified in Figures 2C and 2D. The protease-mediated inhibition of SRP-dependent integration of op-156 (triangles) correlated with the digestion of Sec61α (circles) but not SRβ (squares). Glycosylation of op-156 by the V_{200}-PK-RM could not be reconstituted with SRα (data not shown). These data suggest that proteolysis of Sec61α is the primary cause for the observed defects in protein translocation.

Nascent Polypeptides Are Not Transferred to Sec61α in Protease-Inactivated PK-RM

To define which stage in the translocation reaction is compromised by protease digestion of the PK-RM, we used the amine reactive cross-linker dissucinimidylsuberate (DSS) to identify translocation components that are in contact with a nascent polypeptide (pG64) derived by translation of a truncated VSV G mRNA. As depicted in Figure 3A, radiolabeled pG64 will be cross-linked to SRP54 if the signal sequence remains bound to SRP (Figures 3Aa–3Ac). If the signal sequence is transferred from SRP54 to a novel protein X or the membrane surface (Figure 3Ad), we should observe reduced cross-linking of pG64 to SRP54, and we might detect cross-links between pG64 and protein X. Insertion of pG64 into the translocation channel (Figure 3Ae) should likewise reduce cross-linking to SRP54 and yield cross-links to Sec61α or protease-derived fragments of Sec61α. Intermediates c and d are not detected when intact microsomes are assayed in the presence of GTP, because the RNC is rapidly transferred from SRP54 to the Sec61 complex upon GTP binding to SRP54 and SRα (Rapiejko and Gilmore, 1997).

A truncated mRNA encoding pG64 was translated in a wheat germ system in the presence or absence of SRP to prepare SRP-RNCs and RNCs. DSS-mediated cross-linking of pG64 to SRP54 and Sec61α yields the cross-linked products designated as SRP54* and Sec61α* (Kellaris et al., 1991; Rapiejko and Gilmore, 1997). In accordance with accumulation of intermediates a and b in Figure 3A, pG64 was cross-linked to SRP54 but not Sec61α when microsomes or GTP were deleted from the assays (Figure 3B). Additional control experiments confirmed that formation of Sec61α* was dependent upon the inclusion of DSS, SRP, and active microsomes (e.g., T_0-PK-RM). The absence of the SRP* product in assays containing GTP and T_0-PK-RM indicates that the Sec61 translocation channels are present in excess relative to the SRP-RNCs. When the T_X-PK-RM were reconstituted with SRα, cross-links between pG64 and Sec61α were most intense in assays of T_1-PK-RM, barely detectable with T_7-PK-RM, and undetectable with T_{20}-PK-RM (Figure 3B). Although the absence of intact Sec61α in the T_7-PK-RM and T_{20}-PK-RM (Figure 1A) readily explains the absence of intact Sec61α*, further examination of the autoradiogram failed to disclose novel products that could correspond to pG64 cross-linked to Sec61α fragments. When inactive membranes (i.e., T_{20}-PK-RM) were assayed, the intensity of SRP54* was comparable to that observed in control assays that lacked PK-RM or GTP, indicating that SRP54 does not dissociate from the signal sequence of pG64 to yield either of the post SRP-cycle intermediates (Figure 3Ad or 3Ae).

Cross-links between intact Sec61α and pG64 were detected in assays that contained the SRα fragment plus mildly digested C_X-PK-RM (Figure 3C). When more extensively digested membranes were assayed (e.g., C_{50}-PK-RM), pG64 was primarily cross-linked to SRP54. As observed with the T_X-PK-RM, reduced formation of Sec61α* was accompanied by a corresponding increase in cross-linking of pG64 to SRP54. Fractionation of cross-linking assays over alkaline sucrose gradients showed that the product designated as SRP54* was primarily recovered in the supernatant fraction and Sec61α* was recovered in the membrane pellet fraction. These cross-linking experiments indicate that an SRP-cycle intermediate (Figures 3Aa, 3Ab, or 3Ac) accumulates in assays of the SRα reconstituted membranes that display translocation defects (e.g., T_{20}-PK-RM or C_{50}-PK-RM). In several cross-linking experiments, we

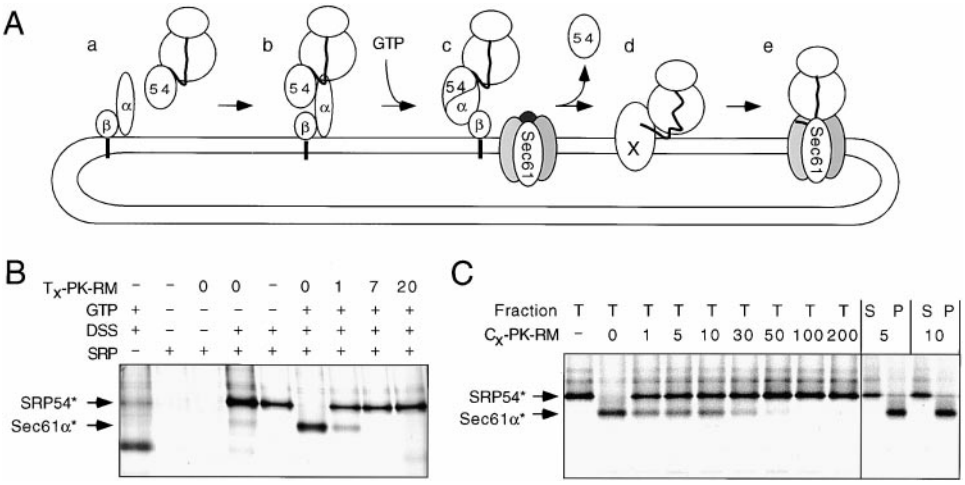

Figure 3. Cross-Linking of Nascent Chains to Sec61α in Protease-Digested PK-RM

(A) Sequential intermediates (a–e) in translocation across protease-digested PK-RM can be assigned by DSS-mediated cross-linking of a nascent polypeptide (pG64) to SRP54 (a–c), a putative protein X (d), or to Sec61α or Sec61α fragments (e).

(B and C) Six eq of T_X-PK-RM or C_X-PK-RM were reconstituted with the 1.2 pmol of the SRα fragment (C) or with in vitro translated SRα (B). RNCs or SRP-RNCs bearing pG64 assembled in a wheat germ system were applied to S-200 gel filtration columns to remove GTP. The GTP-depleted SRP-RNCs and the RNCs derived from 7.5 μl of translation products were mixed with T_X-PK-RM (B) or C_X-PK-RM (C) in the presence of 100 μM GTP, except as noted. After a 20 min incubation at 25°C, DSS was added to cross-link pG64 to adjacent proteins. In (C), the cross-linking assays were either processed directly for PAGE in SDS (T) or were subjected to Na_2CO_3 fractionation to obtain supernatant (S) or membrane pellet (P) fractions. Cross-linked products between radiolabeled pG64 and SRP54 or Sec61α are designated as SRP54* and Sec61α*. The cross-linked product that comigrates with SRP54 in the left-hand lane of (B) is thought to be pG64 cross-linked to wheat germ SRP54.

detected a faint product that is derived by cross-linking of pG64 to Sec61α severed in cytoplasmic loop 8 by chymotrypsin. A cross-link between pG64 and Sec61α severed in cytoplasmic loop 6 was not detected.

GTP Binding to the SR-SRP-RNC Complex

To discriminate between the initial two intermediates (a and b) and the posttargeting intermediate (c) depicted in Figure 4A, we next asked whether SRP and SRα bind GTP when an SRP-RNC is targeted to the SRα-reconstituted C_XPK-RM or T_XPK-RM. To address this question, we modified a well characterized assay to monitor the formation of Gpp(NH)p stabilized complexes between SRP and ^{35}S-methionine-labeled SRα (Rapiejko and Gilmore, 1992, 1997). The protease-digested PK-RM were reconstituted with in vitro translated SRα and separated from GTP, unincorporated SRα, and the majority of the reticulocyte lysate SRP by gel filtration chromatography. SRP-RNCs bearing pG64 were separated from GTP and unbound SRP by centrifugation. These two preparations were combined in the presence or absence of Gpp(NH)p to permit the assembly of SRP-SR complexes upon targeting of the SRP-RNCs to the membrane-bound SR. Formation of a Gpp(NH)p stabilized SRP-SR complex can be detected by cosedimentation of radiolabeled SRα with SRP on a sucrose density gradient in detergent solution (Figure 4B, circles). Efficient formation of Gpp(NH)p stabilized SRP-SR complexes was observed for T_1-PK-RM, C_{50}-PK-RM, and V_{200}-PK-RM when SRP-RNCs were present (Figure 4C). In contrast, the T_{20}-PK-RM have a reduced capacity to form SRP-SR complexes (Figure 4C). Control experiments were performed using RNCs or mock-assembled SRP-RNCs obtained by translation of a protein that lacks a signal sequence (firefly luciferase, ffLuc77). These controls show that trace amounts of reticulocyte lysate SRP are responsible for the residual assembly of SRP-SR complexes by the T_{20}-PK-RM. We conclude that translocation of proteins across the trypsin-digested membranes (e.g., T_{20}-PK-RM) is blocked prior to GTP binding to SRP54 and SRα (intermediates a or b). Translocation of proteins across the C_{50}-PK-RM and V_{200}-PK-RM is blocked after GTP binding to SRP54 and SRα (intermediate c).

We next asked whether the translocation intermediates were membrane bound (Figures 4Ab, 4Ac, or 4Ae) or soluble (Figures 4Aa or 4Ad). SRP-RNCs bearing pG64 were incubated with PK-RM in the absence or presence of GTP prior to chromatography on gel filtration columns equilibrated in buffers containing 150 mM KOAc (L), 300 mM KOAc (M), or 500 mM KOAc (H) to resolve free and membrane-bound RNCs (Figure 4D). In the presence of GTP, the RNC is transferred to the Sec61 complex and elutes with the microsomes in a low- or high-salt buffer (Figure 4D). In the absence of GTP, fewer RNCs were membrane bound, and this interaction was sensitive to hypertonic solutions. Binding of SRP-RNCs to the SRα-reconstituted microsomes was analyzed in the presence of GTP on columns equilibrated in 150 mM KOAc (Figure 4E). Compared to the undigested control membranes (T_0-PK-RM), the trypsin-digested membranes that lack intact SRβ (T_{20}-PK-RM) are defective in targeting of the SRP-RNC to the SRα reconstituted microsomes (Figure 4E). The protease-digested PK-RM that display normal GTP binding to SRα and SRP54 (T_1-PK-RM, C_{50}-PK-RM or V_{200}-PK-RM) accumulate membrane bound RNCs. In the absence of SRα, RNC binding

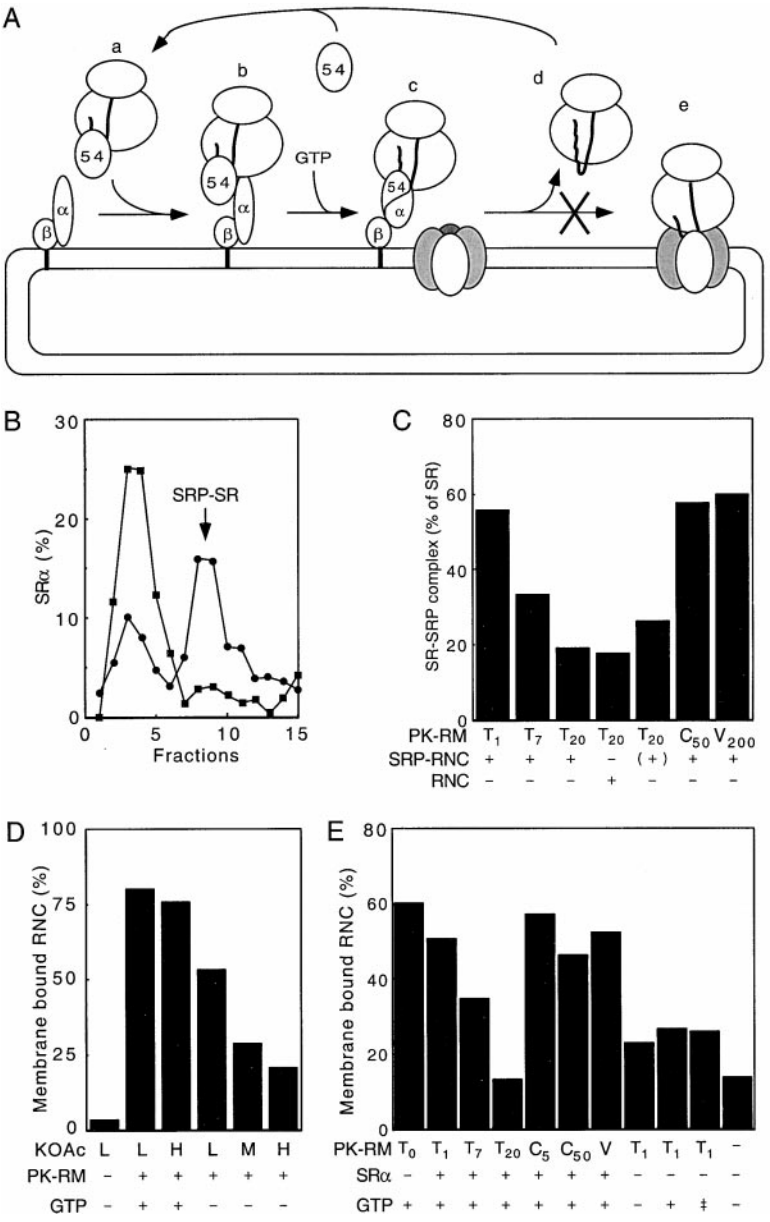

Figure 4. Formation of Complexes between SR and SRP-RNCs

(A) Four translocation intermediates (a–d) are depicted that might accumulate if transfer of the RNC from SRP54 to Sec61α (e) is blocked.

(B–E) RNCs and SRP-RNCs bearing PG64 were assembled as described in Figure 3.

(B and C) The formation of Gpp(NH)p stabilized SRP-SR complexes was assayed as described in the Experimental Procedures using purified SRP-RNCs as the source of SRP and protease-digested PK-RM bearing ^{35}S methionine-labeled SRα.

(B) The SRα-reconstituted T_1-PK-RM were combined with SRP-RNCs in the absence (squares) or presence (circles) of 100 μM Gpp(NH)p in an isotonic buffer (50 mM TEA, 150 mM KOAc, 2.5 mM $Mg(OAc)_2$, 1 mM DTT, and 0.002% Nikkol). Detergent solubilized SRP-SR complexes (fractions 7–12) were resolved from free SRα (fractions 1–6) by sucrose density centrifugation.

(C) The percentage of SRα that cosediments with SRP in a Gpp(NH)p-dependent manner was calculated from gradients similar to that shown in (B). The SRP-RNC sample in parentheses was from a translation programmed with ffLuc77 mRNA.

(D and E) SRP-RNCs bearing pG64 were separated from GTP by S-200 gel filtration chromatography. Aliquots of the SRP-RNCs derived from 10 μl of translation products were incubated with 4 eq of PK-RM (D) or 4 eq of protease-digested PK-RM (T_x-PK-RM, C_x-PK-RM or V_{200}-PK-RM ["V" in E]) plus 600 fmol of the SRα fragment (E). The assays contained 100 μM GTP (+), 100 μM Gpp(NH)p (double dagger), or no nucleotide (-). Membrane-bound RNCs were separated from unbound RNCs by gel filtration chromatography on 1 ml sepharose CL-2B columns equilibrated in 50 mM TEA, 2.5 mM $Mg(OAc)_2$, and 1 mM DTT plus either 150 (L), 300 (M), or 500 (H) mM KOAc. The percentage of the pG64 that eluted with the microsomes was quantified after PAGE in SDS.

to the T_1-PK-RM was reduced nearly to background levels, thereby showing that this assay primarily monitors SRP-dependent targeting of RNCs to the membrane. The residual binding of RNCs to T_1-PK-RM observed in the absence of SRα was nucleotide independent (Figure 4E) and salt insensitive (data not shown), consistent with SRP-independent attachment of RNCs to the Sec61 complex. Taken together with the results presented in Figures 3 and 4C, we can conclude that the inactive SRα reconstituted membranes that retain intact SRβ (e.g., C_{50}-PK-RM or V_{200}-PK-RM) accumulate a novel posttargeting intermediate (Figure 4Ac) when signal sequence transfer to the Sec61 complex is blocked. Our results are not compatible with signal sequence dissociation from SRP54 upon GTP binding to the SRP-SR complex in membranes that lack an active Sec61 complex (Figure 4Ad), unless SRP rebinds to the signal sequence to initiate another targeting cycle. The latter possibility will be addressed in Figure 6.

Sec61-Dependent Release of the Signal Sequence from SRP54

The purified SRP receptor was reconstituted into liposomes to provide a model system to examine SR function in the absence of the Sec61 complex. Binding of SRP-RNCs bearing radiolabeled pG64 to the SR proteoliposomes was evaluated using gel filtration columns equilibrated in 150 mM KOAc or 300 mM KOAc to discriminate between the targeting and posttargeting intermediates. As SRP-SR complexes are relatively stable in 300 mM KOAc in the presence of Gpp(NH)p, but not GDP (Connolly et al., 1991), this assay should reveal whether the posttargeting intermediate is formed in the absence of the Sec61 complex. SRP-RNCs do not bind

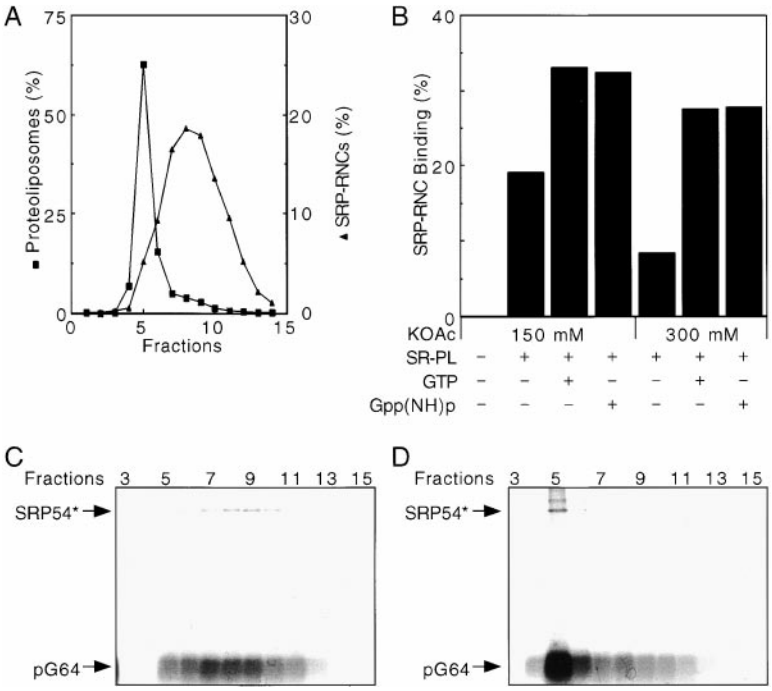

Figure 5. Targeting of SRP-RNCs to SR Proteoliposomes

SR proteoliposomes and control liposomes lacking SR were prepared as described in the Experimental Procedures. The pG64 mRNA was translated in the presence of 60 nM SRP to prepare SRP-RNCs that were separated from GTP as in Figure 4D. SRP-RNCs derived from 2 μl of translation products were incubated with SR proteoliposomes (~1 pmol SR) or control liposomes for 20 min at 25°C in the absence or presence of 100 μM GTP or 100 μM Gpp(NH)p prior to chromatography on sepharose CL-2B columns equilibrated in 50 mM TEA, 5.0 mM $Mg(OAc)_2$, and 1 mM DTT plus either 150 mM KOAc (A, B, C, and D) or 300 mM KOAc ([B], as noted).

(A) The elution profile of SR proteoliposomes containing ^3H phosphatidylcholine (squares) was determined by scintillation counting. Eluate fractions containing pG64-RNCs (triangles) were spotted onto Whatmann 3MM filter paper, precipitated in cold 10% TCA, and boiled in 5% TCA prior to scintillation counting.

(B) Proteoliposome-bound and free SRP-RNCs were resolved as in (A). The percentage of SR proteoliposome–bound SRP-RNCs is shown after subtracting as background the 14% of pG64 that elutes in fractions 4–6 in the absence of SR.

(C and D) SRP-RNCs derived from 15 μl of translation products were incubated with control liposomes (C) or SR proteoliposomes ([D], ~6 pmol of SR) in the presence of 100 μM GTP. Proteoliposome-bound RNCs were resolved from unbound RNCs as in (A). DSS was added to the eluted fractions to cross-link pG64 to SRP54.

to control liposomes that lack SR; hence, these unbound SRP-RNCs (Figure 5A, triangles) elute later than SR proteoliposomes (Figure 5A, squares). In the absence of GTP, 18% of the SRP-RNCs eluted with the SR proteoliposomes in the physiological ionic strength buffer (Figure 5B), diagnostic of targeting intermediate formation as depicted in Figure 4Ab. If GTP binding to SRP54 and SRα promotes signal sequence dissociation from SRP54, we should observe a drastic reduction in RNC binding to the SR proteoliposomes. Instead, both GTP and Gpp(NH)p increased the amount of RNCs that eluted with the SR proteoliposomes at 150 mM KOAc. In the absence of GTP, SRP-RNC binding to the SR proteoliposomes was reduced 2.3-fold when the ionic strength was raised to 300 mM KOAc. In the presence of GTP or Gpp(NH)p, the proteoliposome-bound RNCs were 2-fold less sensitive to the increased ionic strength, indicating that both GTP and Gpp(NH)p stabilize the proteoliposome-bound SRP-RNC.

According to our model for the posttargeting intermediate, SRP54 should remain bound to pG64 when the RNC is attached to the SR proteoliposome. Alternatively, if RNC attachment to the SR proteoliposome can be mediated by an interaction between SRβ and the ribosome (Bacher et al., 1999), GTP binding to SRP54 and SRα should promote dissociation of SRP54 from the signal sequence. A cross-linking assay was used to discriminate between these alternatives. The majority of the pG64-RNCs coeluted with the SR proteoliposomes in the presence of GTP (Figure 5D). More importantly, treatment of the proteoliposome-bound RNCs with DSS yielded the SRP54* cross-linked product. Notably, the intensity of the SRP54* was not reduced after incubation with the SR proteoliposomes (Figure 5D) relative to control liposomes (Figure 5C). Cross-linking of pG64 to SRP54 was substantially reduced when the SRP-RNCs were incubated with proteoliposomes that contain both the SR and the Sec61 complex (data not shown). In agreement with a previous report (Görlich and Rapoport, 1993), we observed that translocation of preprolactin across the SR-Sec61 complex proteoliposomes was less efficient than across PK-RM; hence, other RER membrane or lumenal proteins may be required to achieve robust translocation across the proteoliposomes.

We tested the possibility that multiple GTP binding and hydrolysis cycles by the SRP-SR complex could account for the persistent binding of SRP54 to the RNC when Sec61α was severed in loops 6 and 8 (cycling through intermediates a-d in Figure 4A). If this explanation is correct, we should observe a decrease in the yield of pG64 cross-linked to SRP54 if the SRP is unable to rebind to the RNC after the initial targeting cycle has been completed. To test this hypothesis, we repeated the cross-linking experiment shown in Figure 3C with two modifications designed to prevent rebinding of SRP to the RNC (Figure 6). The pG64 mRNA was translated in the wheat germ system to assemble SRP-RNCs, which were subsequently purified by centrifugation to remove any unbound SRP. The SRP-RNCs were then incubated with the SR-reconstituted microsomes in the presence of Gpp(NH)p to prevent dissociation of the SRP-SR complex (Connolly et al., 1991), thereby preventing rebinding of the SRP to the RNC. When the SR is present in excess

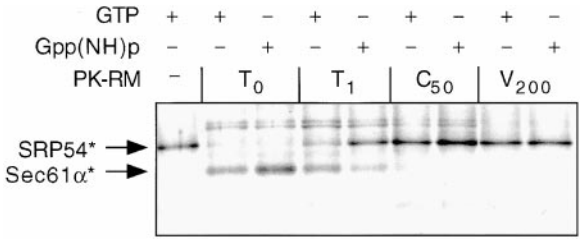

Figure 6. Persistent Binding of SRP54 to the Signal Sequence of a Membrane-Bound RNC

The pG64 mRNA was translated in the presence of 60 nM SRP to assemble SRP-RNCs that were separated from free SRP by centrifugation through a high-salt cushion. The SRP-RNC complexes derived from 7.5 μl of translation products were mixed with 8 eq of T_X-PK-RM, C_{50}-PK-RM, or V_{200}-PK-RM, and 1 pmol of the SRα fragment in the presence of 100 μM GTP or 100 μM Gpp(NH)p. After a 20 min incubation at 25°C, DSS was added to cross-link pG64 to SRP54 or Sec61α. Radiolabeled polypeptides that correspond to SRP54* and Sec61α* were resolved by PAGE in SDS.

relative to the SRP-RNCs (e.g., T_0-PK-RM), the RNC is quantitatively transferred from SRP to the Sec61 complex in assays that contain GTP or Gpp(NH)p. When we assayed microsomes that lack intact Sec61α (C_{50}-PK-RM or V_{200}-PK-RM), the intensity of the SRP54* product was not reduced in assays that contained Gpp(NH)p relative to control assays that contain GTP or assays that lack microsomes. These results fully support the view that the signal sequence does not dissociate from SRP54 in the absence of the intact Sec61 complex.

Discussion

Proteolysis of SRα and SRβ

We have used proteolytic digestions of ribosome-stripped microsomes to sever cytoplasmic domains of the SR, the TRAM protein, and the Sec61 complex. As observed previously, proteolysis of SRα blocks protein translocation prior to targeting of the SRP-RNC to the microsome. Reconstitution of the protease-digested PK-RM with exogenous SRα allowed the detection of two additional protease-induced blocks in protein translocation that are caused by inactivation of SRβ or Sec61α. Digestion of PK-RM with moderate amounts of trypsin (e.g., T_{20}-PK-RM) yielded membranes that bind the SRα fragment, despite the lack of intact SRβ. Upon reconstitution with the SRα fragment, the trypsin-digested membranes remain defective in targeting of the SRP-RNC to the SRP receptor. Further work will be required to elucidate the role of SRβ during the initial interaction between the SRP-RNC and the SR.

Proteolysis of the Sec61 Complex

Digestion with the three proteases shown here, as well as with thermolysin (data not shown), revealed a hierarchy of proteolytic sensitivity of Sec61α cytoplasmic segments. Cytoplasmic loop 8 and the C-terminal tail were most readily severed, followed by loop 6. The N terminus of Sec61α was considerably less sensitive, while cleavage within loops 2 and 4 was never detected. Proteases with different cleavage specificities yielded remarkably similar digestion products, suggesting that the protease cleavage sites in Sec61α are tightly clustered on surface-exposed loops. The N-terminal segment of *S. cerevisiae* Sec61p is proposed to be embedded on the cytoplasmic surface of the RER membrane as an amphipathic α helix (Wilkinson et al., 1996). Canine Sec61α may adopt a similar conformation, which could explain the protease resistance of the N terminus of canine Sec61α.

Proteolysis of Sec61α was accompanied by a striking decrease in SRP-dependent translocation. A previous report indicates that chymotrypsin digestion of PK-RM severs Sec61α and blocks a posttargeting stage of the translocation reaction (Murphy et al., 1997). Although the protease-induced intermediate detected by Murphy et al. was not extensively characterized, it is likely identical to that described here. Incomplete digestion of Sec61α (e.g., C_5-PK-RM) resulted in a partial loss of activity that correlated closely with residual intact Sec61α. The close correlation between translocation activity and residual intact Sec61α is best explained by the hypothesis that Sec61 oligomers have impaired SRP-dependent translocation activity when one or two Sec61α subunits are severed.

Recent systematic photo-cross-linking studies have shown that the signal sequence of a nascent polypeptide contacts TM spans 2 and 7 of yeast Sec61p (Plath et al., 1998). Proteolysis of Sec61α in loop 6 will separate TM span 7 from the N-terminal half of Sec61α. Pairs of complementary N- and C-terminal fragments of *S. cerevisiae* Sec61p have been tested for the ability to complement a *sec61* null mutation (Wilkinson et al., 1997). With the exception of N-terminal fragments terminating in loops 6 or 7, coexpression of the complementary C-terminal fragment yielded a viable strain in the absence of intact Sec61p. Thus, an intact cytoplasmic loop 6 is a crucial element for Sec61α function.

Inactivation of Sec61α Causes Accumulation of Membrane-Bound SRP-RNCs

In the absence of GTP, targeting of an SRP-RNC to the RER results in a salt-sensitive interaction between the SRP-RNC and the SR (Connolly and Gilmore, 1986). With intact microsomes, GTP binding to SRP54 and SRα promotes the rapid transfer of the signal peptide from SRP54 to a vacant Sec61 complex. Consequently, the posttargeting intermediate had not been detected in our previous studies. When Sec61α is inactivated by proteolysis or deleted from proteoliposomes, the interaction between the SRP-RNC and the SR is stabilized by GTP or Gpp(NH)p binding to SRα and SRP54 and prolonged by the lack of an acceptor for the RNC. Although the GTP-stabilized association between the SRP-RNC and the SR proteoliposome suggests that GTP hydrolysis by SRP54 and SRα is also inhibited, the latter conclusion is in conflict with the results of GTPase assays that have been conducted by Bacher et al. (1996, 1999) using RNCs, SR proteoliposomes, and SRP. Further studies will be required to determine which of the three GTPases (SRα, SRβ or SRP54) are activated under these circumstances.

When assayed in detergent solution, the purified SR initiates the GTP-dependent dissociation of SRP from the RNC (Connolly and Gilmore, 1989). Hence, one might

have predicted that GTP binding to the SRP-SR complex would induce efficient signal sequence dissociation from SRP54 despite the absence of the Sec61 complex in SR proteoliposomes or protease-digested microsomes. As neither GTP nor Gpp(NH)p causes a decrease in SRP54 cross-linking to the nascent polypeptide, we can conclude that dissociation of SRP54 from the RNC occurs when two conditions are satisfied. The first condition is satisfied when both SRP54 and SRα bind GTP in a cooperative reaction (Rapiejko and Gilmore, 1997). The second condition is met by the presence of an acceptor for the signal sequence, which is provided by the Sec61 complex. We hypothesize that detergent micelles serve as an artificial acceptor for the signal sequence when the SR is assayed in detergent solution. Although SR proteoliposomes allow a partial release of the SRP-mediated translation arrest (Görlich and Rapoport, 1993), the 5-fold excess of SR relative to SRP in the latter assays may have interfered with the initial binding of SRP54 to the signal sequence.

A recent report (Murphy et al., 1997) describes a post-targeting intermediate that accumulates when in vitro translocation assays contain an excess of SRP-RNCs relative to accessible Sec61 complexes. Differential salt extraction experiments revealed two distinct populations of membrane-bound RNCs. One population corresponded to RNCs that were attached to the Sec61 complex while a second population was proposed to be attached via a membrane component, either protein or phospholipid, that interacts with the signal sequence prior to insertion into the Sec61 complex (Murphy et al., 1997). The circumstance that leads to accumulation of the latter intermediate may not be fundamentally different from inactivation of the Sec61 complex by proteolysis or deletion of the Sec61 complex from proteoliposomes.

Regulation of the SRP-SR GTP Hydrolysis Cycle by the Sec61 Complex

The demonstration that dissociation of the signal sequence from SRP54 is inhibited in the absence of a functional protein translocation channel suggests that the Sec61 complex regulates the GTPase cycle of the SRP-SR complex at the stage of signal sequence dissociation from the SRP. We propose that regulation of the GTPase cycle of the SRP-SR complex by Sec61 provides a mechanism to insure that the signal sequence is directly inserted into a translocation channel upon release from SRP54. This hypothesis implies a direct physical interaction between the Sec61 complex and either the SR or the SRP-RNC at a stage prior to signal sequence dissociation from SRP54. This regulatory scheme could provide a mechanism to discriminate between Sec61 complexes that are currently engaged in protein translocation, and hence occupied by an RNC, and Sec61 complexes that are either vacant or occupied by a nontranslating ribosome. The regions of Sec61α that are exposed on the cytoplasmic face of the membrane are logical sites for an interaction with the SRP-RNC or SR. A comparison of eleven eukaryotic Sec61α sequences reveals that loops 6 and 8 are more highly conserved with respect to the number and location of charged amino acid residues than the N- and C-terminal tails and cytoplasmic loop 2. Cytoplasmic loops 6 and 8 of Sec61α are candidates for a region of the Sec61 complex that might interact with the SRP-SR complex.

Experimental Procedures

Preparation of SRP, SR, Protease-Digested PK-RM, and the 52 kDa SRα Fragment

Rough microsomes (RM), KOAc-washed rough microsomes (K-RM), and SRP were isolated from canine pancreas as described (Walter et al., 1981). The SR was purified from K-RM as described (Connolly and Gilmore, 1993). K-RM were digested for 1 hr at 4°C with elastase (0.5 μg/ml) to generate the 52 kDa SRα fragment that was purified as described (Nicchitta and Blobel, 1989).

Puromycin-high salt extracted rough microsomes (PK-RM) were prepared from RM as described (Raden and Gilmore, 1998), except that the PK-RM were washed once by centrifugation rather than twice with 50 mM triethanolamine-acetate, pH 7.5 (50 mM TEA), 600 mM KOAc, 12 mM $Mg(OAc)_2$, 1 mM DTT, and 1 mM EDTA. The PK-RM were resuspended in 50 mM TEA, 250 mM sucrose, and 1 mM DTT at a concentration of 2 eq/μl (eq as defined in Walter et al., 1981). 500 μl aliquots of the PK-RM were digested with trypsin (0–30 μg/ml) or chymotrypsin (0–200 μg/ml) on ice for 1 hr at a PK-RM concentration of 2 eq/μl. Digestion with endoproteinase Glu-C (200 μg/ml) was for 1 hr at 37°C. Trypsin and chymotrypsin digestions were terminated by a 15 min incubation with 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by adjustment to 10 μg/ml of aprotinin. Digestions with endoproteinase Glu-C were terminated by adjustment to 1 mM 3,4 dichloroisocoumarin. The protease-digested PK-RM were adjusted to 550 mM KOAc and centrifuged for 30 min at 100,000 g in a Beckman Type 50 rotor. The membranes were resuspended at a concentration of 0.1 eq/μl in 250 mM sucrose, 50 mM TEA, and 1 mM DTT, and centrifuged for 30 min at 100,000 g in a Beckman Type 50 rotor. After repeating the preceding centrifugation step, the protease-digested PK-RM were stored at −80°C at a concentration of 1 eq/μl in 250 mM sucrose, 50 mM TEA, and 1 mM DTT.

Translocation Assays of Protease-Digested PK-RM

The mRNAs encoding SRα, preprolactin, and N-terminal truncation products of opsin (op156), VSV G protein (pG64), firefly luciferase (ffLuc77), and Sec61α (Sec61α274 and Sec61α393) were isolated from preparative-scale transcriptions as described (Rapiejko and Gilmore, 1992). DNA templates for transcription of the Sec61α truncation products (Sec61α274 and Sec61α393) were prepared by PCR-mediated amplification of the Sec61α pSPUTK plasmid (Knight and High, 1998).

Protease-digested PK-RM were assayed in the absence of SRα by translating preprolactin mRNA in a reticulocyte lysate reaction that was supplemented with T_X-PK-RM, C_X-PK-RM, or V_X-PK-RM. To provide exogenous SRα, 25 μl translocation assays that contained 1.2 eq of protease-digested PK-RM were supplemented with 200 fmol of the SRα 52 kDa fragment.

The procedure to assay GTP-dependent integration of op156 into protease-digested microsomes has been described previously (Rapiejko and Gilmore, 1992). Aliquots of ribonucleotide-depleted SRP-RNCs prepared by translation of op156 in a reticulocyte lysate system were incubated for 30 min at 25°C with 1.2 eq of T_X-PK-RM, C_X-PK-RM, or V_X-PK-RM plus 200 fmol of the SRα fragment in the presence or absence of GTP.

Preparation of SR Proteoliposomes

SR proteoliposomes were prepared using a modification of a previously described method (Görlich and Rapoport, 1993). Purified SR (5 pmol), bovine phosphatidylcholine (35 nmol, Sigma P-7763), and bovine serum albumin (10 μg) were combined in a total volume of 10 μl of 20 mM HEPES-KOH (pH 7.5), 350 mM KOAc, 1 mM DTT, and 0.1% Nikkol. The SR proteoliposomes formed during a 15 hr incubation with 10 mg of Bio-Beads SM-2 were recovered by centrifugation as described (Görlich and Rapoport, 1993).

DSS Cross-Linking

The pG64 mRNA was translated in a wheat germ system in the absence or presence of 60 nM SRP to prepare RNCs or SRP-RNCs. Aliquots of the pG64 RNCs were incubated at 25°C with T_x-PK-RM, C_x-PK-RM, or V_x-PK-RM that were reconstituted with in vitro translated SRα or the 52 kDa SRα fragment. After 20 min, the reactions were adjusted to 400 μM DSS using a freshly prepared stock solution of DSS in dimethyl sulfoxide to cross-link the radiolabeled pG64 to SRP54 or Sec61α. After a 5 min incubation at 25°C, the cross-linking reactions were quenched by adjustment to 100 mM glycine (pH 8.7).

SRP-SR Complex Formation Assay

The SRP-SR complex formation assay using SRP-RNCs as the source of SRP is based upon our previous method (Rapiejko and Gilmore, 1992). The protease-digested PK-RM were reconstituted with in vitro translated ^{35}S methionine-labeled SRα as described (Rapiejko and Gilmore, 1992). Free NTPs were removed from the reconstituted microsomes bearing ^{35}S methionine-labeled SRα by chromatography on 1 ml sepharose CL-2B columns equilibrated in 50 mM TEA, 150 mM KOAc, 5 mM $Mg(OAc)_2$, 0.002% Nikkol, and 3 mM DTT. To prepare SRP-RNCs, the pG64 mRNA transcript was translated at 25°C for 15 min in a wheat germ reaction with 60 nM SRP but lacking ^{35}S methionine. The SRP-RNCs were separated from unbound SRP by centrifugation through a high-salt sucrose cushion (Raden and Gilmore, 1998). Aliquots of the SRα-reconstituted microsomes were incubated for 20 min at 25°C with the purified SRP-RNCs in the presence of 100 μM GTP or Gpp(NH)p, adjusted to 300 mM KOAc, and solubilized for sucrose density gradient centrifugation to separate free SR from SRP-SR complexes (Rapiejko and Gilmore, 1992). Radioactive SRα in the gradient fractions was quantified after PAGE in SDS. The percentage of Gpp(NH)p stabilized SRP-SR complex = 100 × (% SRα in fractions 7–12) / [(% SRα in fractions 1–6) + (% SRα in fractions 7–12)] after subtracting as background the percentage of SRα that was recovered in fractions 7–12 in the presence of GTP.

Protein Immunoblots

The procedure for protein immunoblots using ECL has been described (Raden and Gilmore, 1998). Multiple film exposures were obtained to insure that the ECL signal was linear with respect to the quantity of antigen. A rabbit polyclonal antibody was raised against the C terminus of Sec61α (KEQSEVGSMGALLF) using standard procedures.

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